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Influence of Altitude Variation on Trigonelline Content during Ontogeny of *Coffea Canephora* Fruit

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Abstract

Trigonelline is a nicotinic acid related alkaloid abundant in coffee beans. The influence of altitude variations on trigonelline and its precursor nicotinic acid content of beans of Robusta coffee plants that collected from different elevations were investigated. Both nicotinic acid and trigonelline contents were always higher at all stages of coffee fruit ontogeny from plants grown at higher elevations. Maximum nicotinic acid and trigonelline content of 5.164 ± 0.131 mg and 975.8 ± 7.24 mg/100 g Fresh weight respectively was evident in stage V seeds (9 months DAF) of high altitude collected samples.

Keywords: Trigonelline, Robusta, Ontogeny, Nicotinic acid, Elevation

1. Introduction

Coffee is a rich source of bioactive metabolites such as caffeine, trigonelline, chlorogenic acid, arabinogalactans, melanoidins along with ash, organic acids, and caffeic acid etc and their profile in beans is important and helpful to know the quality of coffee brew (Sridevi & Giridhar, 2008). Among these caffeine, CGAs and trigonelline are major compounds which imparts bitterness in coffee, apart from their diverse physiological functions in humans (Clifford & Wilson, 1985). Intake of such bioactive compounds from coffee drink may be associated with either health benefits or risks. Coffee consumption has been correlated to reduced risk of colon rectal cancer (Lee, Inouem, Otani, Iwasaki, Sasazuki & Tsugane, 2007), Type 2 diabetes (Campos & Baylin, 2007) and Alzheimer's disease (Barranco, Allam, Serrano Del Castillo & Fernandez, 2007). Recent studies demonstrated caffeine's positive effects such as pshychoactive response and neurological condition such as Parkinson disease infant hyperactivity, and metabolic disorder like diabetic gallstones and liver function (Dorea & da Costa, 2005). Similarly, coffee bean contains trigonelline (N-methylnicotinic acid or N-methylbetaine of pyridine-3-carboxylic acid) as the second abundant alkaloid compound and it thermally converted to nicotinic acid (antipellagra factor) and some flavour compounds during roasting (Taguchi, 1988). In addition it is considered important for taste and nutrition (Adrian & Fragne, 1991).

Trigonelline content in green coffee beans is in the range of 0.88 to 1.77% in Arabica, 0.75 to 1.24% in Robusta coffee (Ky, Doulbeau, Guyot, Chareir, Hamon, Louarn & Noirot, 2000a), 1.02% in *C. pseudozangaeboriae* and 0.57% of DMB in *C. liberica* var Deweveri (Ky, Guyot, Louran, Hamon, & Noirot, 2000b) respectively. Several health promoting properties such as hypoglycaemic, hypocholesterolemic, antitumor, antimigraine, or antiseptic effects have been attributed to triogonelline as cited in Allred, Yackley, Vanamala and Allred (2009). As a biomarker, trigonelline contains inherent measurable biological activity and is a unique compound with many diverse properties. Several studies have attributed the anticancer properties of coffee to metabolites other than caffeine (Hirose, Niwa, Wakai, Matsio, Nakanishi & Tajma, 2007) and the same was further strengthened by a recent study which showed phytoestrogenic nature of trigonelline from coffee beans (Allred *et al.*, 2009).

Trigonellline is synthesized by the methylation of nicotinic acid by S-adenosyl-L-Methionine (SAM) dependent nicotinate N-Methyltransferase (EC 2.1.1.7) which has been found in crude extracts of pea (Joshi & Handler, 1960) and in coffee leaves (Taguchi, Yamaki Sakaguchi & Shimabayashi, 1987). Trigonelline and its synthetic ability from nicotinic acid are distributed in all parts of coffee seedlings (Zheng & Ashihara, 2004; Mazzafera, 1991). However the levels of nicotinic acid are quite less compared to trigonelline as most of the nicotinic acid is converted to trigonelline. In raw coffee beans NA is in the range of 16-44 µg/g (Hughes & Smith, 1946; Carvalho,1962; Casal, Olivera, & Ferreira, 2000). During roasting trigonelline partially degrades to produce pyridines and nicotinic acid and other minor compounds which impart flavour and aroma, hence essential from organoleptic features point of view. Various studies indicate that trigonelline demethylating enzyme activity leads to nicotinic acid production (Taguchi & Shimabayashi, 1983) and also by pyrolysis of trigonelline (Viani & Horman, 1974). The demand for high quality coffee is rapidly increased over the last few

years and is still expected to increase. Though arabica coffee brewed from beans of *C. arabica* is preferred as filter coffee, robusta coffee from *C. canephora* too is having importance in global market as it is used as soluble coffee (instant coffee).

The altitude at which coffee is grown plays a major role in determining the quality of the bean, because there is less oxygen, coffees grown at higher altitudes take longer to mature than plants grown at lower altitudes. This allows the flavours to develop more fully and produces beans that are delicate and flavourful. The levels of trigonelline may be influenced by various extrinsic and intrinsic factors, expected to vary geographically and also the impact of environment cannot be ruled out. In India, though both arabica and Robusta are under cultivation, robusta coffee is often blended with Arabica. In the present study investigations were made to analyse the changes in trigonelline and nicotinic acid content in beans of *Coffea canephora* (robusta) that harvested from plants grown at different altitudes.

2. Materials and methods

Fruits of *C. canephora* P. ex Fr. CxR variety were collected from plantations grown at different elevations near Mudigere of Chikamagalur District of Karnataka during February 2009. Ripened fruits from plants grown at higher (3700 ft MSL), medium (3300 ft MSL) and lower altitudes (3000 ft MSL) were collected from different places viz., Devaramane, Guthi (Javali) and Tripura respectively (Lat 13° 7' 60N, Long 75° 37' 60E). At least ten random plants were selected at each of the said altitude, and at least 250g of fresh fruits were collected from these plants. These samples were immediately taken into sterile polythene bags with small perforations and used for experiment within 24 hours.

Extraction of trigonelline and nicotinic acid

To determine trigonelline contents beans from ripened fruits of five different stages were harvested i.e. Stage I (3 months DAF), Stage II (5 months DAF), Stage III (7 months DAF), Stage IV (8 months DAF) and Stage V (10 months DAF). These stages roughly represent pinhead stage immediately after dormancy period, rapid expansion stage, pericarp growth stage, efficient endosperm formation stage and lastly dry matter accumulation stage to harvesting stage respectively (Koshiro, Zheng, Wang, Nagai & Ashihara, 2006). Fruit pulp was removed and green beans were taken out. The seeds were allowed to dry to attain ~12-15% moisture content. All the green beans were then grounded finely. To 5 g powder 25 ml water was added, and the mixture was autoclaved for 20 minutes at 120º C (Taguchi, Sakaguchi & Shimabayashi, 1985). After cooling, the sample was centrifuged for 10 minutes at 1,300 xg. To the precipitate, 5 ml of water was added, and the well-mixed solution was centrifuged again before the supernatant was collected. The extraction procedure and washing the precipitate was conducted twice more and the supernatants were combined. All the extracts were pooled and filtered (0.2µm. pore size filter) and directly analysed by HPLC.

HPLC analysis

HPLC analysis was performed on Shimadzu LC 20 A (Shimadzu Corp., Kyoto, Japan) equipped with CLASS-VP integrator software for data processing. A C-18, 5.0 µm, 4.6 mm x 250 mm column (Sunfire column of Waters, USA) was used. The solvent system used was

methanol: water (3:1 v/v), and the flow rate was 1 ml/min. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 268 nm. The compounds were identified by their retention times, chromatographic comparisons with authentic standards, and their UV spectra. Quantification was based on the external standard method. Under the assay conditions described, a linear relationship between the concentration and the UV absorbance was obtained at 268 nm for trigonelline and nicotinic acid (Casal *et al.,* 2000).

Statistical Analysis

Five coffee bean samples, out of the ten samples each that were randomly collected from coffee plants grown at higher, medium and lower elevations were extracted and analysed for trigonelline and nicotinic acid. The samples were extracted and analysed in five replicates. Values from all five replicate determinations of each sample were averaged and represented as means with standard deviations. Data were analysed statistically by the SPSS 17.0 software by Two-way ANOVA and homogenous subsets were determined to separate the mean values of the different altitudes and developmental stages. Means with statically significantly difference (different subsets) was marked with different alphabetical letters. Linear regression was calculated for nicotinic acid and trigonelline, with elevation as the independent variable.

3. Results and Discussion

There was significant variations in trigonelline (Trig) and nicotinic acid (NA) levels of green beans of fruits collected from plants grown at different altitudes. The Trig levels were positively influenced by altitude variation, with a maximum of (975.8 ± 7.24 mg/ 100 g) in beans of high altitude samples, which was 20% and 25% more than that of medium and lower altitudes respectively (Figure 1). Similarly there was significant variation observed in both Trig and NA during the ontogeny of coffee bean development (Figure 1, 2). The levels of NA too were positively influenced by altitude variation, with a maximum of (5.16 mg/ 100 g) in beans of high altitude samples (Figure 2). In stage I samples (3 months DAF) i.e. immediately after dormant period, wherein tiny fruits appear, the NA and Trig contents were (0.686 ± 0.078 mg) to 1.25 ± 0.068 mg) and (20.86 ± 3.23 to 32.4 ± 4.75 mg/ 100 g) respectively at low to high altitudes and in subsequent stages of bean development there was a gradient increase in both NA and Trig content and it reached to (3.92 ± 0.127 mg to 5.164 ± 0.131 mg/100g) and (734.8 ± 7.39 to 975 ± 7.24 mg /100 g) at harvesting stage of samples collected from low and high altitudes respectively. Trigonelline concentration at all stages of fruit development increased significantly with increasing elevation (Figure 3). The regression was significant (y=0.1739x-217.17), with high coefficient of determination as R^2 = 0.9774. Similarly, nicotinic acid concentration too increased significantly with increasing elevation, wherein regression was y=0.0015x-2.6174 with coefficient of determination as R^2 = 0.8526 which is slightly less than that of trigonelline (Figure 3). In stage V fully ripened fruit's bean trigonelline concentration regression too was significant (y=0.3509x-336.53) with high coefficient of determination as R^2 =0.9434 which is almost same to R^2 values of average trigonelline content of at all stages of ontogeny. Even for nicotinic acid of stage V fruit beans, the coefficient of determination (R^2 = 0.8729) was similar to that of ontogeny

stages (R^2 = 0.8526). So in the present study, the way altitude (quantitative variable) affect the secondary metabolite trigonelline and also its precursor nicotinic acid was interpreted through linear correlation. A glance at nicotinic acid profile during ontogeny shows that, there was significant variation at stage II fruit, stage III, IV and V seed of medium altitude (mean = 2.35, 3.964, 4.108, 4.634; standard deviation = 0.367, 0.149, 0.291, 0.268 respectively), compared to low and high altitudes, which indicates the influence medium altitude was prominent for nicotinic acid. Similar observations were noticed for trigonelline too.

In the present study, the extraction and HPLC analysis of both Trig and NA facilitated to obtain the data significantly ($p < 0.05$). To validate the efficiency of extraction procedure one sample was extracted three times and the coefficient of variation was 1.54% and 1.12% for Tirgonelline (Trig) and nicotinic acid (NA). Similarly a linear relationship between Trig / NA concentration and UV absorbance was perceived, wherein the linearity was managed over the concentration range of (0.15-400 µg/mL and 0.10-400 µg/mL) and the correlation coefficient for the Trig/NA standard curve invariably exceeded 0.999. In HPLC the retention time (RT) for NA and Trig were 2.03 and 2.58 respectively (Figure 4). In case of seed sample extracts another major peak was evident at 4.54 RT. This peak is nothing but caffeine of seed extracts and data of caffeine is out of scope of this study as it requires different extraction method and also absorption maxima (274 nm).

In the present study, there was significant variation in Trig and NA content in developing seeds during ontogeny of *C. canephora* fruits. The pyridine alkaloid Trig accumulates in seeds, however its biosynthetic activity is reported to be higher in the pericarp (Koshiro *et al.*, 2006). Nicotinic acid being the precursor for Trig production, the Trig data in the present study at all stages of ontogeny of fruit was in direct relation to NA levels. A decreasing trend in Trig content of pericarp was evident when the fruit becomes mature and the rate of reduction was more from stage III to stage V fruits. The trend was almost same in samples collected from plants grown at different altitudes. In stage III pericarp more trigonelline was detected compared to earlier stage of growth. In *C. canephora* in general the fruit development requires 8 to 9 months and it is to some extent asynchronous. This may be one of the reasons for variation in bioactives content in beans during ontogeny of fruit. However, a tendency for synchrony was observed during the later stages of maturation when a significantly higher proportion of fruits entered the largest sized ripe 'cherry' stage as opined (De Castro, Estanislau, Carvalho & Hilhorst, 2005). Trigonelline was well documented from various plants (Poulton, 1981; Barz, 1979; 1985) and trigonelline is normally synthesized in almost all parts of coffee plant (Zheng, Nagai & Ashihara 2004), and its accumulation is higher in young tissues as reported by Zheng and Ashihara (2004). Zheng *et al.* also reported trigonelline activity in endosperm stage. Our results indicate that though Trig was found in significant amounts in pericarp at initial growth stages of fruit, its accumulation was more evident in beans at maturation stage of fruit, which was supported by earlier studies of Zheng *et al.* in *C. Arabica*, wherein, high net biosynthetic activity of Trig was demonstrated in dry matter accumulation stage of seeds i.e. stage III - IV, which subsequently decreases markedly,

however, Trig transportation takes place from pericarp to seeds. A similar observation for caffeine too was showed (Baumann & Wanner, 1972) for *C. arabica.*

Longer maturation times and increased bean sizes are usually observed for plants grown at high altitude or in shade conditions (Muschler, 2001; Guyot, Gueule, Manez, Perriot, Giron & Villain, 1996). A positive relations between altitude and taster preferences of Arabica coffee from different terroirs of Costa Rica was reported by Avelino, Barboza, Araya, Fonseca, Davrieux, Guyot and Cilas (2005). The increase in trigonelline content of robusta beans from high altitude in our study was further supported by earlier report of Avelino et al. (2005). Though the slope exposure whrere in, the light or shade availability too have some influence on Arabica coffee quality as opined by Avelino et al. (2005), in the present study, altitude influence only was investigated as it mainly influence biochemical profile of robusta beans (Guyot et al. 1996). Apart from this, in the present investigation, the range of altitude difference from where samples were collected was 3000-3700 ft and this mesoclimate conditions influence was found to be significant on both Trig and NA content of beans. In contrast to the study of Avelino et al. (2005), negative relation between elevation and triogonelline content was found by Bertrand, Vaast, Alpizar, Etienne, Davrieux and Charmetnat (2006), but this study is based on Near-Infrared Spectra (NIR) of some Arabica hybrids involving Sudanese-Ethiopian origins with traditional varieties. Such variations in metabolic profiles trend is a common phenomenon as it is indirectly influenced by some environmental and coffee agroforestry systems (Vaast et al. 2006). Similarly, roasting conditions and brewing methods too influence the metabolic profile of beans (Sridevi, Giridhar & Ravishankar, 2011). Unlike other metabolites of Coffee, Trig is important from post-harvest processing aspects that imparts quality to Coffee. Because Trig alkaloids gives rise to many flavour (aroma) compounds, like alkyl-pyradines and pyrroles (De Maria, Trugo, Moreira & Werneck 1994). In view of this, the levels of Trig in developing seeds are important though we don't have any information about the actual role of this in developing fruits unlike polyamines and free diterpenes (Sridevi, Giridhar & Ravishankar, 2009, 2010). Shimizu and Mazafera (2000) showed the role of trigonelline during inhibition and germination of coffee seeds. Though Trig production takes place in pericarp of young fruits, the same will decline in pericarp of ripened fruits (Koshiro *et al.,* 2006). However, our data suggests that trigonelline accumulation takes place in seeds from stage III to stage V fruits. This was in concurrence with earlier studies (Zheng *et al.,* 2004; Koshiro *et al.,* 2006). All these substantiate the trend in Trig content and also its precursor nicotinic acid content in the present study. Moreover the content of Trig in harvested beans is on par with its trend in *C. canephora* as reported earlier which would be in the range of (0.75 to 1.24%) as reviewed by De Castro and Marraccini (2006). Certainly, the altitudes at which coffee plants grow had influence on Trig and NA content.

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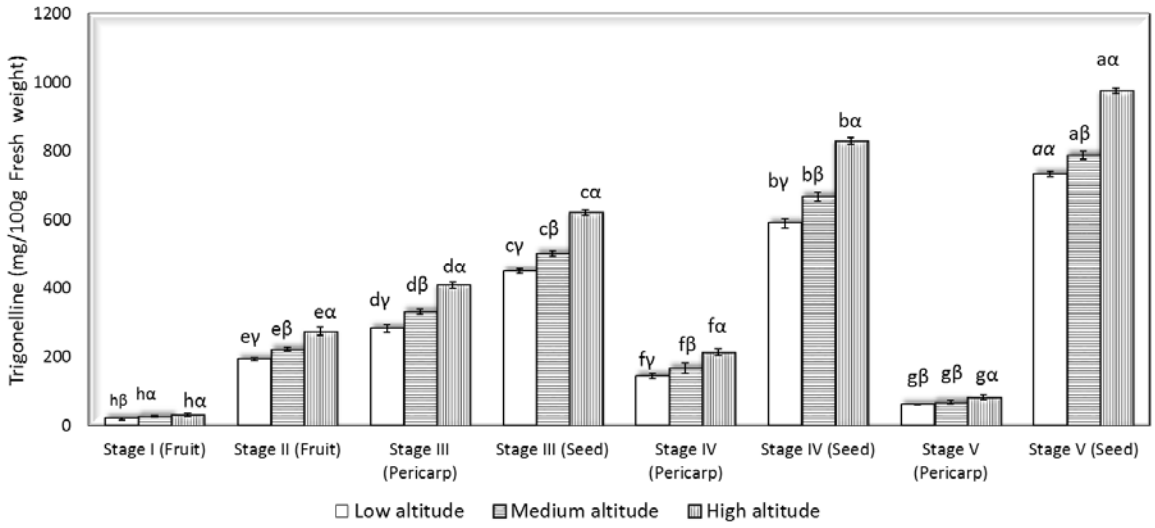

Figure 1. Trigonelline (mg/100g Fresh weight) profiles in beans of *C.canephoara* CxR variety during ontogeny of fruit (values are mean ± SD of five analyses); Different alphabet letters indicate the statistical significant difference within the different developmental stages ($p< 0.05$), and different symbols (α, β and γ) represent statistical significant difference within the altitude ($p< 0.05$).

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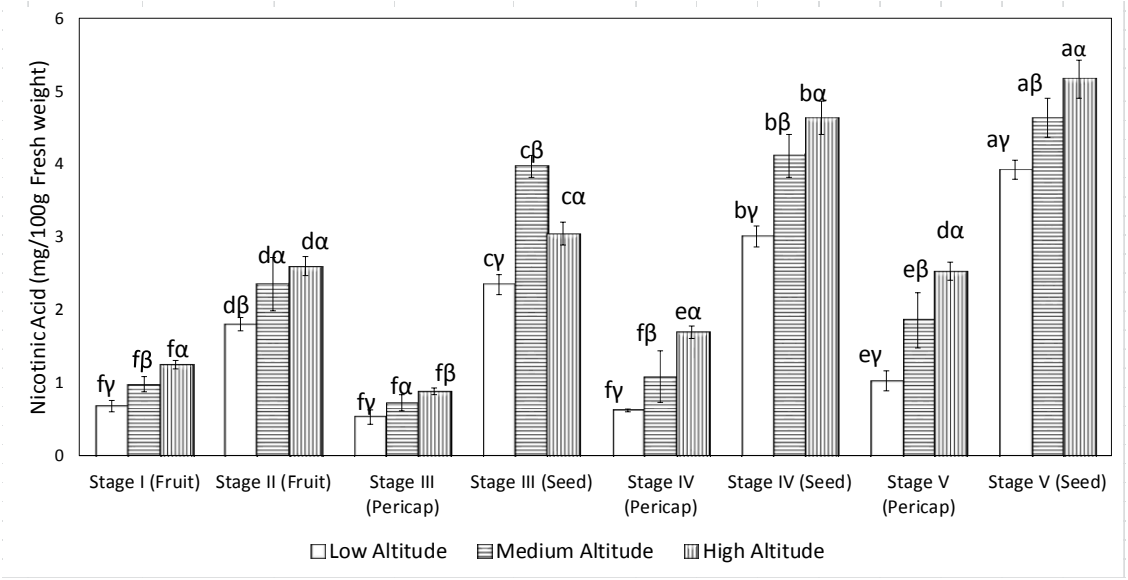

Figure 2. Nicotinic acid (mg/100g Fresh weight) profiles in beans of *C.canephoara* CxR variety during ontogeny of fruit (values are mean ± SD of five analyses); Different alphabet letters indicate the statistical significant difference within the different developmental stages ($p< 0.05$), and different symbols (α, β and γ) represent statistical significant difference within the altitude ($p< 0.05$).

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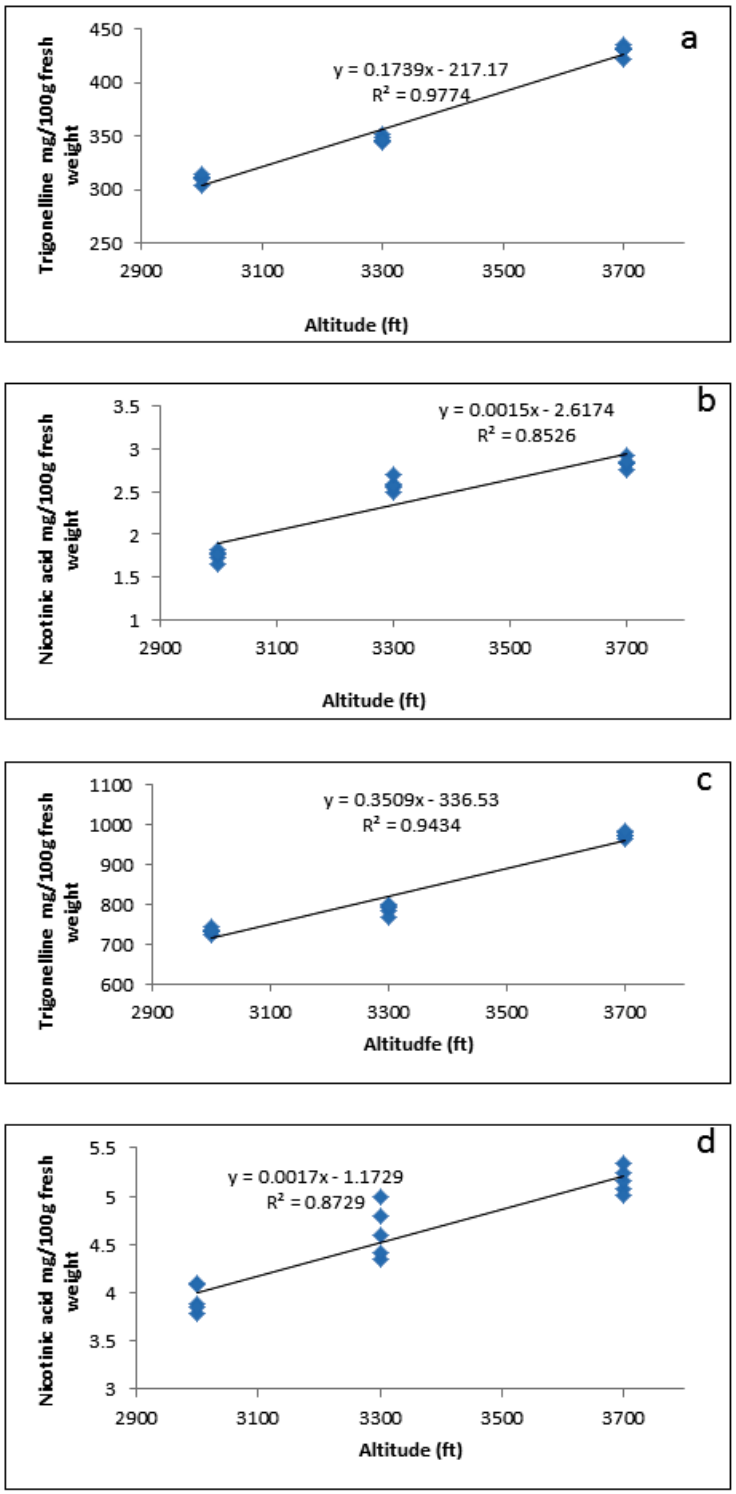

Figure 3. Regression coefficients for effect of elevation on a) variation in trigonelline content during ontogeny of *C. canephora* fruit, b) variation in nicotinic acid content during ontogeny of *C. canephora* fruit, c) variation in stage V seed trigonelline content, d) variation in stage V seed nicotinic acid content

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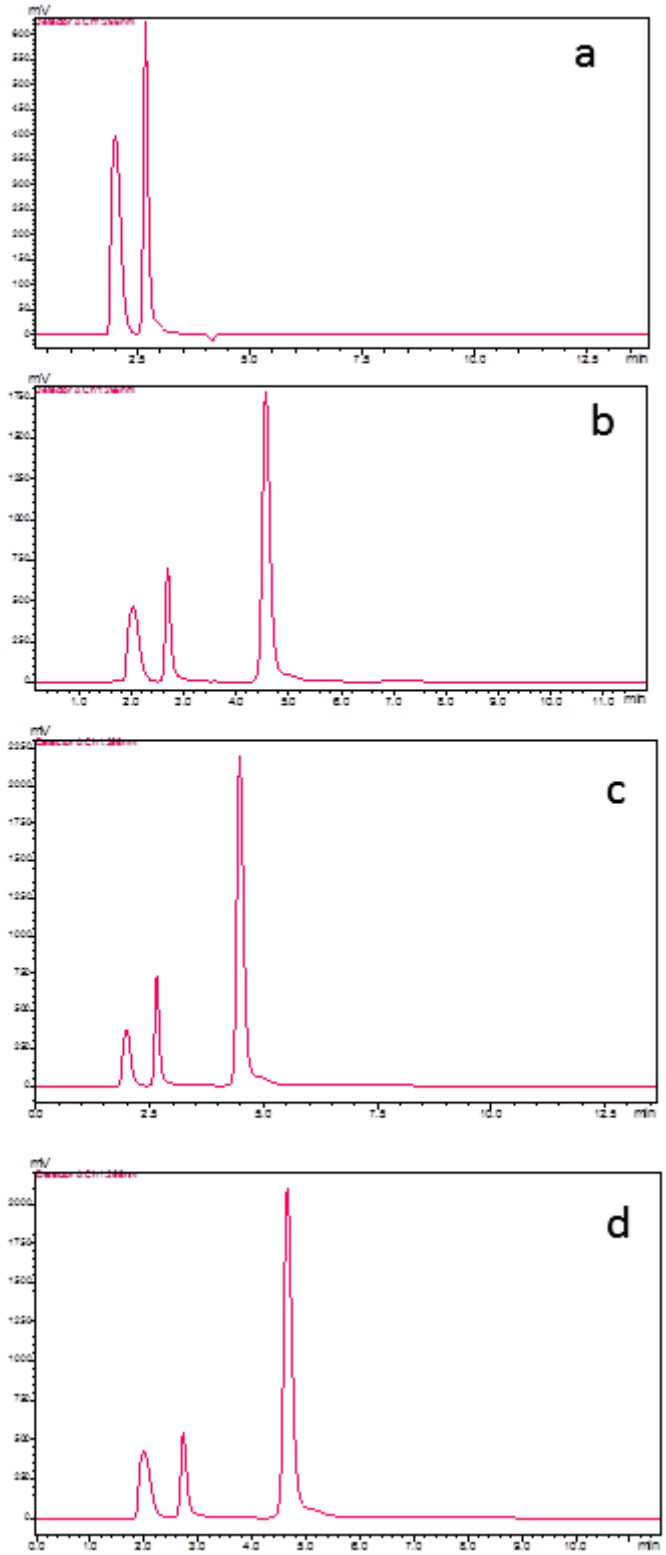

Figure 4. HPLC chromatogram of nicotinic acid (RT 1.990) and trigonelline (RT 2.663) in seed extracts of *C. canephora* fruit grown at different altitudes; a) standards b) Lower altitude sample c) middle altitude sample d) higher altitude sample